= inlet

Fig.

stabilising. hybridisation immunising p. 10-15

Helicobacter felis vaccine

Field of the Invention

The present invention relates to novel *Helicobacter* urease subunit polypeptides, nucleic acid sequences encoding these polypeptides, to the polypeptides for use in vaccines and to the use in the manufacturing thereof, to vaccines comprising said polypeptides and to methods for the preparation of such vaccines. Further, the invention relates to diagnostic methods for the detection of the nucleic acid sequences, the polypeptides and antibodies against the polypeptides.

Background of the Invention

Several *Helicobacter* species are the cause of pathogenesis of the gastric epithelium. *Helicobacter pylori*, and to a lesser extent *H. heilmannii* are known to cause gastritis, a major factor in the development of peptic ulcers and gastric lymphoma in humans. *Helicobacter felis* is most likely the cause of gastric infections in both cats and dogs. In order to survive the highly acidic environment of the stomach, members of the *Helicobacter* family produce ab urease that is capable of hydrolysing the urea present in gastric juice. This hydrolysation sets free an amount of NH_4OH that suffices to neutralise the environment of the bacterium. It is known that the urease plays a role in the colonization of the bacterium as well as in its pathogenesis.

Genes encoding urease have been described and sequenced for both *Helicobacter pylori* (Labigne et al., J. Bacteriol. 173: 1920-1931 (1991)) and *Helicobacter felis* (Ferrero et al., Molec. Microbiol. 9, 323-333 (1993)). Of the seven genes involved in urease expression and secretion, only two genes encode the two structural subunits urease A ~~or~~ and B of the urease enzyme, ureA and ureB. These two polypeptides form a polypeptide complex having urease activity.

Vaccines against infections caused by both *H. pylori* and *felis* have been made and have been the subject of i.a. International Patent Applications WO 94/09823 and WO 96/34624. Several attempt have been made, to use *H. pylori* urease as a vaccine component for the protection of cats against *H. felis* infection. Although indeed a certain level of protection can be obtained, the results are far from the 100 % protection that would be desirable. From animal experiments published so far, it becomes clear that a significant number of animals vaccinated with *H. pylori* is not at all protected against subsequent challenge with *H. felis*. Protection of cats vaccinated with purified urease from either *H. felis* or *pylori* has not been described. Vaccinating cats with *H. felis* whole cell lysates might theoretically be feasible but is not a practical option. This is because in spite of many attempts for improvement, *H. felis* is difficult to grow.

There clearly is a need for an efficacious vaccine, based upon homologous components, and it is clear that the known *H. felis* urease does not confer full protection.

Summary of the Invention

It is i.a. an object of the present invention to provide a *H. felis* urease which is able to induce protection against *Helicobacter felis* infection in dogs and cats. It was surprisingly found ~~now~~ that in *H. felis* a second urease exists, of which the genes encoding the structural subunits share only low homology with the known *H. felis* ureA and B genes. The novel urease is named ureaseXY, in order to discriminate it from the known urease AB. The newly found urease has been discovered in *H. felis*, and is not present in *H. pylori*.

Detailed Description of the Invention

The overall genetic structure of the genes encoding the two structural urease subunits, UreX and UreY, is comparable to that of the known UreA and B in *H. felis* and *H. pylori*. The sequence homology is however surprisingly low. It was even more surprisingly found, that the homology between the ureA and B genes and the novel ureX and Y genes in one single *H. felis* strain is even strikingly lower than the homology between the various ureA and B genes from the various *Helicobacter* species.

Insert (a) from p. 14

Tables 1a, 1b and 1c show the comparison of the ureX and Y genes and the polypeptides they encode from five different *Helicobacter felis* species, with the ureA and B genes and polypeptides from *Helicobacter felis*, *pylori* and *heilmannii*.

The level of homology of the genes encoding the novel structural urease subunits X and Y and the polypeptides they encode as compared to that of known ureA and B genes and polypeptide subunits is presented in Table 1a, b and c.

Reference molecule : *H. felis* ureX CS1	a.a.	n.a.
H. felis ureA	50 %	57 %
H. pylori ureA	52 %	60 %
H. heilmannii ureA	54 %	62 %
H. felis strain Kukka ureX	100 %	91 %
H. felis strain Ds4 ureX	99 %	91 %
H. felis strain 2301 ureX	99 %	91 %
H. felis strain 390 ureX	99 %	91 %

Table 1a: amino acid and nucleic acid homology between the *H. felis* ureX and various ureA subunits.

Reference molecule : *H. felis* ureY CS1	a.a.	n.a.
H. felis ureB	73 %	71 %
H. pylori ureB	73 %	70 %
H. heilmannii ureB	74 %	71 %
H. felis strain Kukka ureY	99 %	95 %
H. felis strain Ds4 ureY	98 %	94 %
H. felis strain 2301 ureY	99 %	95 %

Table 1b: amino acid and nucleic acid homology between the *H. felis* ureY and various ureB subunits.

Reference molecule: *H. felis* ureXY CSI	n.a.
H. felis ureAB	67 %
H. pylori ureAB	67 %
H. heilmannii ureAB	68 %
H. felis strain Kukka ureXY	94 %
H. felis strain Ds4 ureXY	94 %
H. felis strain 2301 ureXY	94 %

Table 1c: nucleic acid homology between *H. felis* ureXY and various ureAB genes.

One embodiment of the invention thus relates to nucleic acid sequences encoding the novel urease X and Y subunits.

First of all, this embodiment of the invention relates to nucleic acid sequences encoding two subunits of a urease complex such as expressed by *Helicobacter felis*, ~~that~~ have at least 85 % homology with SEQ ID NO: 1, or parts thereof with a length of at least 40, preferably 45, more preferably 50 nucleotides encoding at least an immunogenic fragment of one of the subunits. Still even longer fragments, with a length of at least 55, 60 or 70 ~~nucleic acids~~ nucleotides are in that order even more preferred.

A preferred form of this embodiment relates to nucleic acid sequences that encode the urease X subunit polypeptide or the urease Y subunit polypeptide and that have at least 85 % homology with SEQ ID NO: 1, or parts thereof with a length of at least 40, preferably 45, more preferably 50 nucleotides encoding at least an immunogenic fragment of the urease X subunit polypeptide or the urease Y subunit polypeptide. Merely as an example: the nucleic acid sequence encoding the urease X subunit of *Helicobacter felis* strain CS1 starts at position 206/207/208 (GTG) (See figure 1a (1)) and stops at position 884/885/886 (TAA). The nucleic acid sequence encoding the urease Y subunit of *Helicobacter felis* strain CS1 starts at position 897/898/899 (ATG) and stops at position 2601/2602/2603 (TAG). Nucleotides
Still even longer fragments, with a length of at least 55, 60 or 70 ~~nucleic acids~~ are in that order even more preferred.

A more preferred form of this embodiment relates to nucleic acid sequences having at least 90 %, preferably 94 %, more preferably 97 % homology with SEQ ID NO: 1.

The determination of the homology percentages was done with the computer program Align Plus for Windows, available from Scientific and Educational Software, P.O.Box 72045 Durham, NC 27722-2045, USA. Settings used for the nucleic acid comparisons are indicated in figures 1a, 1b and 1c.

Since the present invention discloses nucleic acid sequences encoding novel structural *Helicobacter felis* urease subunits, it is now for the first time possible to obtain such polypeptides in sufficient quantities. This can e.g. be done by using expression systems to express the genes encoding the UreX and UreY subunits.
Therefore, in a more preferred embodiment, the invention relates to DNA fragments comprising a nucleic acid sequence according to the invention. Such DNA fragments can e.g. be plasmids, into which a nucleic acid sequence according to the invention is cloned. Such DNA fragments are useful e.g. for enhancing the amount of DNA for use as a probe, as described below.

An essential requirement for the expression of the nucleic acid sequence is an adequate promoter operably linked to the nucleic acid sequence. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression.
Therefore, an even more preferred form of this embodiment relates to a recombinant DNA molecule comprising a DNA fragment or a nucleic acid sequence according to the invention that is placed under the control of a functionally linked promotor. This can be obtained by means of e.g. standard molecular biology techniques. (Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

Functionally linked promotors are promotors that are capable of controlling the transcription of the nucleic acid sequences to which they are linked. When the host cells are bacteria, useful expression control sequences which may be used include the Trp promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J., 1, 771-775, 1982); the bacteriophage lambda promoters and operators (Remaut, E. et al., Nucl. Acids Res., 11, 4677-4688, 1983); the α-amylase (B. subtilis) promoter and operator, termination sequences and other expression enhancement and control sequences compatible with the selected host cell.

When the host cell is yeast, useful expression control sequences include, e.g., α-mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983) or the metallothionein promoter (Brinster, R.L., Nature, 296, 39-42, 1982) or a heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985).

Bacterial, yeast, fungal, insect and mammalian cell expression systems are very frequently used systems. Such systems are well-known in the art and generally available, e.g. commercially through Clontech Laboratories, Inc. 4030 Fabian Way, Palo Alto, California 94303-4607, USA. Next to these expression systems, parasite-based expression systems are very attractive expression systems. Such systems are e.g. described in the French Patent Application with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W.: Public. Date 1 December 1993).

Thus a still even more preferred form of this embodiment of the invention relates to Live Recombinant Carrier micro-organisms (LRCs) comprising a gene encoding the UreX or UreY polypeptide or an immunogenic fragment thereof according to the invention. Such micro-organisms are e.g. bacteria and viruses. These LRC micro-organisms are micro-organisms in which additional genetic information, in this case a gene encoding the UreX or UreY polypeptide or an immunogenic fragment thereof according to the invention has been cloned. Animals infected with such LRCs will produce an immunogenic response not only against the immunogens of the vector, but also against the immunogenic parts of the polypeptide(s) for which the genetic code is additionally cloned into the LRC, e.g. the ureX or Y gene.

As an example of bacterial LRCs, attenuated Salmonella strains known in the art can attractively be used.

Live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998))

Also, LRC viruses may be used as a way of transporting the nucleic acid sequence into a target cell. Live recombinant carrier viruses are also called vector viruses. The site of integration of the gene encoding a UreX or Y polypeptide may be a site in a viral gene that is not essential to the virus, or a site in an intergenic region. Viruses often used as vectors are Vaccinia viruses (Panicali et al; Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E.P.A. 0473210A2), and Retroviruses (Valerio, D. et al; in Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), Experimental Haematology today - 1988. Springer Verlag, New York: pp. 92-99 (1989)).

The technique of *in vivo* homologous recombination, well-known in the art, can be used to introduce a recombinant nucleic acid sequence into the genome of a bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid sequence according to the invention in the host animal.

Finally another form of this embodiment of the invention relates to a host cell comprising a nucleic acid sequence encoding a polypeptide according to the invention, a DNA fragment comprising such a nucleic acid sequence or a recombinant DNA molecule comprising such a nucleic acid sequence under the control of a functionally linked promotor. This form also relates to a host cell containing a live recombinant carrier containing a nucleic acid molecule encoding a UreX or Y polypeptide or an immunogenic fragment thereof according to the invention.

A host cell may be a cell of bacterial origin, e.g. Escherichia coli, Bacillus subtilus and Lactobacillus species, in combination with bacteria-based plasmids as pBR322, or bacterial expression vectors as pGEX, or with bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells like insect cells (Luckow et al; Bio-technology 6: 47-55 (1988)) in combination with vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. et al; Cell 32: 1033 (1983), mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

Another embodiment of the invention relates to the polypeptides encoded by the nucleic acid sequences, i.e. the urease X subunit and the urease Y subunit and to immunogenic fragments thereof according to the invention.

Therefore, this embodiment of the invention relates to the *Helicobacter felis* urease X polypeptide, said polypeptide having an amino acid sequence that is at least 85 % homologous to SEQ ID NO: 2 or an immunogenic fragment of that polypeptide with a length of at least 40 amino acids that is capable of inducing an immune response against ureaseXY. Preferably, the length of that fragment is more than 40 amino acids, more preferably at least 45, 50, 55, 60 or 70 amino acids in that order or preference.

Preferably this embodiment relates to such polypeptides having a sequence homology of at least 90 %, more preferably 94 %, even more preferably 97 % homology to SEQ ID NO: 2, or an immunogenic fragment of that polypeptide with a length of at least 40 amino acids, more preferably at least 45, 50, 55, 60 or 70 amino acids in that order or preference that is capable of inducing an immune response against ureaseXY.

This embodiment of the invention also relates to the *Helicobacter felis* urease Y polypeptide, said polypeptide having an amino acid sequence that is at least 85 % homologous to SEQ ID NO: 3 or an immunogenic fragment of that polypeptide with a length of at least 40 amino acids that is capable of inducing an immune response against ureaseXY. Preferably, the length of that fragment is more than 40 amino acids, more preferably at least 45, 50, 55, 60 or 70 amino acids in that order or preference.

Preferably this embodiment relates to such polypeptides having a sequence homology of at least 90 %, more preferably 94 %, even more preferably 97 % homology to SEQ ID NO: 3, or an immunogenic fragment of that polypeptide with a length of at least 40 amino acids, more preferably at least 45, 50, 55, 60 or 70 amino acids in that order or preference that is capable of inducing an immune response against ureaseXY.

As for the nucleotide sequence comparison, the comparison between the various amino acid sequences was made using Align Plus for Windows, available from Scientific and Educational Software, P.O.Box 72045 Durham, NC 27722-2045, USA. Settings used for the amino acid comparisons are indicated in figures 1a, 1b and 1c.

It will be understood that, for the particular polypeptides embraced herein, natural variations can exist between individual *Helicobacter felis* strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath et al in "The Proteins" Academic Press New York (1979). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting polypeptides retain their immunoreactivity. Thus, variations not essentially influencing the immunogenicity of the polypeptide compared to the wild type polypeptide as depicted in SEQ ID NO: 2 or 3 are considered to fall within the scope of the invention. Those variations in the amino acid sequence of a certain structural subunit X or Y according to the invention that still provide a polypeptide capable of inducing an immune response against infection with *H. felis* or at least against the clinical manifestations of the infection are considered as "not essentially influencing the immunogenicity".

When a polypeptide is used for e.g. vaccination purposes or for raising antibodies, it is however not necessary to use the whole polypeptide. It is also possible to use a fragment of that polypeptide that is capable, as such or coupled to a carrier such as e.g. KLH, of inducing an immune response against that polypeptide, a so-called immunogenic fragment. An "immunogenic fragment" is understood to be a fragment of the full-length polypeptide of the structural subunit X or Y that still has retained its capability to induce an immune response in the host, i.e. comprises a B- or T-cell epitope. At this moment, a variety of techniques is available to easily identify DNA fragments encoding antigenic fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent NR. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-4002 (1984), J. Imm. Meth. 102, 259-274 (1987), the so-called PEPSCAN method is an easy to perform, quick and well-established method for the detection of epitopes, the immunologically important regions of the polypeptide. The method is used world-wide and as such well-known to ~~man~~ persons skilled in the art. This (empirical) method is especially suitable for the detection of B-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific polypeptide fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl.

Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzowsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991).

Vaccines against e.g. *Helicobacter pylori*, which has only one urease, can be made on the basis of this urease, as was described above. In the specific case of *Helicobacter felis* however, a vaccine based upon the known *Helicobacter felis* structural subunits ureA and B is not capable of providing sufficient protection against *Helicobacter felis* infection: immunity against structural subunits ureA and B allegedly does not neutralise the urease activity of the newly found heterologous structural subunits UreX and Y.

Therefore, vaccines for the protection of animals against *Helicobacter felis* infection should at least be directed against the novel urease XY.
Therefore, one form of still another embodiment of the invention relates to vaccines capable of protecting mammals such as dogs and cats against *Helicobacter felis* infection, that comprise the structural subunit X or Y, preferably X and Y, more preferably X, Y, A and B, or an immunogenic fragment of X and/or Y according to the invention together with a pharmaceutically acceptable carrier.

Still another embodiment of the present invention relates to the polypeptides according to the invention for use in a vaccine.

Still another embodiment relates to the use of the polypeptide according to the invention in the manufacturing of a vaccine for combating *Helicobacter felis* infections.

One way of making a vaccine according to the invention is by biochemical purification of the ureaseXY polypeptide or its subunits from a bacterial culture. This can e.g. be done by centrifugation of the bacteria, and the use of gel-filtration columns for separation of the urease polypeptide or its subunits from other components. Further purification may e.g. be done by selective precipitation in ammonium-sulphate, followed by centrifugation, gel electrophoresis and, if desired, separation from the urease AB subunits and dissolving the pellet in a suitable buffer. This is however a time-consuming way of making the vaccine, especially where *Helicobacter felis* is difficult to grow.

It is therefore much more convenient to use the expression products of the genes encoding the urease X and Y subunits according to the invention in vaccines. Such vaccines can easily be made by admixing ureaseXY or an UreX or Y subunit or an immunological fragment thereof according to the invention with a pharmaceutically acceptable carrier as described below.

Furthermore, vaccines can comprise live recombinant carriers as described above, capable of expressing ureaseXY, an UreX or UreY subunit or immunogenic fragments thereof according to the invention. Such vaccines, e.g. based upon a Salmonella carrier or a viral carrier infecting the gastric epithelium have the advantage over subunit vaccines that they better mimic the natural way of infection of *Helicobacter felis*.

Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunisation.

Vaccines described above all contribute to active vaccination, i.e. the host's immune system is triggered by the UreX and/or Y polypeptide or immunogenic fragments thereof, to make antibodies against these polypeptides.

Alternatively, such antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the host animal. This method of vaccination, passive vaccination, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for vaccinating immune-compromised animals. Administered antibodies against *Helicobacter* UreX or UreY can in these cases bind directly to the urease excreted by the bacteria. This has the advantage that the urease activity is directly eliminated, thus resulting in acidification of the environment and decreased or stopped *Helicobacter* growth.

Therefore, one other form of this embodiment of the invention relates to vaccines comprising antibodies against *Helicobacter felis* urease X polypeptides that have an amino acid sequence that is at least 85 % homologous to SEQ ID NO: 2 or immunogenic fragments of that polypeptide with a length of at least 40 amino acids that are capable of inducing an immune response against ureaseXY or antibodies against *Helicobacter felis* urease Y polypeptides that have an amino acid sequence that is at least 85 % homologous to SEQ ID NO: 3 or immunogenic fragments of that polypeptide with a length of at least 40 amino acids that are capable of inducing an immune response against ureaseXY.

Vaccines can also be based upon host cells as described above, that comprise ureaseXY, an UreX or UreY subunit or immunogenic fragments thereof according to the invention.

An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding polypeptides has been successful for many different polypeptides. (As reviewed in e.g. Donnelly et al., The Immunologist 2: 20-26 (1993)).

This way of vaccination is very attractive for the vaccination of both cats and dogs against *Helicobacter felis* infection.

Therefore, still other forms of this embodiment of the invention relate to vaccines comprising nucleic acid sequences encoding a polypeptide according to the invention or immunogenic fragments thereof according to the invention, and to vaccines comprising DNA fragments that comprise such nucleic acid sequences.

Still other forms of this embodiment relate to vaccines comprising recombinant DNA molecules according to the invention.

DNA vaccines can easily be administered through intradermal application e.g. using a needleless injector. This way of administration delivers the DNA directly into the cells of the animal to be vaccinated. Amount of DNA in the microgram range between 1 and 100 µg provide very good results.

In a further embodiment, the vaccine according to the present invention also comprises antigens from other dog or cat pathogenic organisms and viruses, or genetic information encoding such antigens. Such organisms and viruses are e.g. Feline Infectious Peritonitis virus, Feline Immune deficiency virus, Canine and Feline Parvovirus,

Distemper virus, Adenovirus, Calicivirus, *Bordetella bronchiseptica, Borrelia burgdorferi, Leptospira interrogans, Chlamydia* and *Bartonella henseli.*

Also, the present invention relates to polypeptides according to the invention for use in the manufacturing of a vaccine for combating *Helicobacter felis* infections.

All vaccines according to the present invention comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Vaccines according to the present invention may in a preferred presentation also contain an adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants are Freunds Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, Quill A(R), mineral oil e.g. Bayol(R) or Markol(R), vegetable oil, and Carbopol(R) (a homopolymer), or Diluvac(R) Forte.

The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the polypeptide adheres, without being covalently bound to it. Often used vehicle compounds are e.g. aluminium hydroxide, -phosphate or -oxide, silica, Kaolin, and Bentonite.

A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span or Tween.

Often, the vaccine is mixed with stabilisers, e.g. to protect degradation-prone polypeptides from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al; J. Bacteriology 59: 509 (1950)), carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

In addition, the vaccine may be suspended in a physiologically acceptable diluent. It goes without saying, that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilising a polypeptide are also embodied in the present invention.

Vaccines according to the invention that comprise the UreX or UreY subunit polypeptide can very suitably be administered in amounts ranging between 1 and 100 micrograms, although smaller doses can in principle be used. A dose exceeding 100 micrograms will, although immunologically very suitable, be less attractive for commercial reasons.

Vaccines based upon live attenuated recombinant carriers, such as the LRC-viruses and bacteria described above can be administered in much lower doses, because they multiply themselves during the infection. Therefore, very suitable amounts would range between 10^3 and 10^9 CFU/PFU for respectively bacteria and viruses.

Many ways of administration can be applied. Intranasal application is a frequently used way of administrating a vaccine. Oral application is also an attractive way of administration, because the infection is often located in the upper digestive tract. A preferred way of oral administration is the packaging of the vaccine in capsules, known and frequently used in the art, that only disintegrate in the highly acidic environment of

the stomach. Also, the vaccine could be mixed with compounds known in the art for temporarily enhancing the pH of the stomach. Systemic application is also suitable, e.g. by intramuscular application of the vaccine. If this route is followed, standard procedures known in the art for systemic application are well-suited.

Another embodiment of the invention relates to diagnostic tests for the detection of *H. felis* infection. It is known that several *Helicobacter* species such as *H. bizzozeronii, H. felis* and *H. salomonis* are capable of infecting both cats and dogs. Of these three, *H. felis* is the species suspected to cause most of the pathology, although it is often outnumbered by *H. bizzozeronii* and *H. salomonis*. Thus, a quick and correct diagnosis of disease, in both cats and dogs, caused by *Helicobacter felis* is important. It has however been very difficult to discriminate between these three species due to the fact that they are so very closely related. Therefore, it is another objective of this invention to provide such diagnostic tools suitable for discriminating *H. felis* from other *Helicobacter* species.

On the basis of the novel urease polypeptides and the genes encoding the urease polypeptides, at least three different diagnostic tests, specifically suitable for the discrimination of *H. felis* from other members of the *Helicobacter* family were developed:
1) a diagnostic test based upon the presence or absence of DNA encoding the specific UreX and UreY structural subunits
2) a diagnostic test based upon the detection of antibodies against the specific UreX and UreY structural subunits
3) a diagnostic test based upon the detection of antigenic material of the specific UreX and UreY structural subunits

A diagnostic test according to 1) is e.g. based upon the reaction of bacterial DNA isolated from the animal to be tested, with specific probes or PCR primers based upon the sequence of ureX or Y genes. If *H. felis* DNA is present in the animal, this will e.g. specifically bind to ureX or Y specific PCR primers and will subsequently become amplified in PCR reaction. The PCR reaction product can then easily be detected in DNA gel electrophoresis.

The DNA can most easily be isolated from the microorganisms present in swabs of the upper digestive tract or in the saliva of the animal to be tested. Specific primers can easily be selected from the many regions of the ureX and ureY coding sequences and the non-coding intergenic sequence that differ in sequence from the comparable regions in the ureAB coding sequences. One of the many algorithms suitable for the determination of the level of nucleic acid homology and for comparison of nucleotide sequences in general is known as "Clustal W". It has been described by Thompson et al., in Nucleic Acid Research 22: 4673-4680 (1994). The program can be found at several sites on Internet. An more recent alternative for this program is e.g. Align Plus for Windows, available from Scientific and Educational Software, P.O.Box 72045 Durham, NC 27722-2045, USA.

As follows from figure 1, a large number of possible PCR primers can be found that are specific for ureX or ureY. An extremely specific pair of PCR probes is e.g. formed by the 5'-located sequence CATGCACTTTTTGAAAAAAGA (SEQ ID NO: 16) and the 3'-located sequence TATGGTGGTCTTCTCT (SEQ ID NO: 17). Of course, many other sequences that are specific for ureX or Y or the intergenic region are suitable. Standard PCR textbooks give methods for determining the suitability of the probes for selective PCR reactions with ureX or ureY. PCR techniques are extensively described in

(Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995)).

Another DNA-based test is based upon growth of bacterial material obtained from the swab, followed by classical DNA purification followed by classical hybridisation with radioactively or colour-labelled ureXY-specific DNA-fragments. Given the very low homology between the ureXY-coding regions and the ureAB coding regions of both *H. felis* and other *Helicobacter* species, hybridisation unambiguously indicates the presence or absence of *H. felis*. Both PCR-reactions and hybridisation reactions are well-known in the art and are i.a. described in Maniatis/Sambrook (Sambrook, J. *et al.* Molecular cloning: a laboratory manual. ISBN 0-87969-309-6).

Selective detection with PCR-primers or with classical hybridisation with ureXY-specific DNA-fragments can be done with fragments that preferably are short, but for practical reasons preferably consist of a stretch of at least 10 contiguous nucleotides of SEQ ID NO: 1. It is clear that for hybridisation experiments a probe needs to be selected that has a higher homology to SEQ ID NO: 1, than to sequences encoding the *Helicobacter* ureA or ureB subunit. Such a probe can very easily be selected with the help of the Align Plus for Windows program or the Clustal W program as discussed above. In a comparative hybridisation experiment the DNA to be diagnosed can be tested next to e.g. H. *pylori* DNA. The probe according to the invention, having a higher homology to SEQ ID NO: 1 than to a gene encoding ureAB, would bind better to *H. felis* DNA (if present in the sample) than to DNA of other *Helicobacter* species, thus specifically revealing the presence of H. *felis* DNA in the sample to be tested. The sequences SEQ ID NO: 16 or 17 mentioned above are merely examples of probes very suitable for labelling and subsequent use in the *H. felis*-specific hybridisation assays as described.

Thus, one embodiment of the invention relates to a diagnostic test for the detection of DNA encoding the specific *Helicobacter* UreX and UreY subunit polypeptides. Such a test comprises a nucleic acid sequence according to the invention or a fragment thereof that is specific for the DNA encoding UreX and UreY or the intergenic region between UreX and UreY. A fragment that is specific for that DNA is a fragment that binds better to the DNA encoding UreX and UreY or the intergenic region between UreX and UreY than to the DNA encoding UreA and UreB or the intergenic region between UreA and UreB.

Methods for the detection of *Helicobacter felis* DNA comprise hybridisation of the DNA to be tested with UreX or Y DNA, or PCR-reaction of the DNA to be tested with UreX or Y DNA specific probes.

A diagnostic test according to 2) for the detection of *Helicobacter felis* antibodies in sera can be e.g. a simple sandwich-ELISA-test in which purified UreX or UreY subunit polypeptides or antigenic fragments thereof according to the invention are coated to the wall of the wells of an ELISA-plate. A method for the detection of such antibodies is e.g. incubation of purified UreX or Y polypeptide with serum from mammals to be tested, followed by e.g. incubation with a labelled antibody against the relevant mammalian antibody. A colour reaction can then reveal the presence or absence of antibodies against *Helicobacter felis* urease XY. Depending on the labelled antibodies used, the selectivity of this system can be improved by pre-incubation of the serum to be tested with urease AB followed by spinning down the precipitate, in order to avoid non-XY-specific reactions.

If antigenic fragments of the UreX or UreY structural subunits according to the invention are used for coating, this pre-incubation step can be skipped.

Another example of a diagnostic test system is e.g. the incubation of a Western blot comprising UreX or UreY polypeptide or an antigenic fragment thereof according to the invention, with serum of mammals to be tested, followed by analysis of the blot. The purified UreX and UreY structural subunits or antigenic fragments thereof according to the invention, suitable for the coating of ELISA plates or for Western blotting can easily be obtained by expression of the ureX and ureY gene as was described by Ferrero for ureA and B (Ferrero et al., Molec. Microbiol. 9, 323-333 (1993)).

Also, the invention relates to methods for the detection in serum of antibodies against *Helicobacter felis* antibodies in which the method comprises the incubation of serum with UreX or UreY polypeptide or an antigenic fragment thereof according to the invention.

A diagnostic test according to 3) based upon the detection of antigenic material of the specific UreX and UreY structural subunits of *Helicobacter felis* antigens and therefore suitable for the detection of *Helicobacter felis* infection can e.g. also be a standard ELISA test. In one example of such a test the walls of the wells of an ELISA plate are coated with antibodies directed against the specific UreX and UreY structural subunits of *Helicobacter felis*. The antigenic material to be tested can if necessary be pre-incubated with antibodies against UreA and B. This will leave the UreX and Y specific epitopes uncovered and therefore the pre-incubated *Helicobacter* species will bind to the ELISA plate only if it comprises UreX or Y, i.e. if it specifically is *Helicobacter felis*. The use of monoclonal antibodies specific for UreX or Y and not reacting with UreA or B are the preferred antibodies in such tests, because they make the pre-incubation step superfluous. Such monoclonal antibodies can easily be obtained by immunising inbred mice with immunising fragments of UreX or Y according to the invention, by techniques also known in the art (See below: Kohler and Milstein).

The polypeptides or immunogenic fragments thereof according to the invention expressed as characterised above can be used to produce antibodies, which may be polyclonal, monospecific or monoclonal (or derivatives thereof). If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are well-known in the art (e.g. Mayer and Walter, eds. *Immunochemical Methods in Cell and Molecular Biology*, Academic Press, London, 1987).

Monoclonal antibodies, reactive against the polypeptide according to the invention (or variants or fragments thereof) according to the present invention, can be prepared by immunising inbred mice by techniques also known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975).

Finally, the invention relates to methods for the detection of antigenic material from *Helicobacter felis* in which the method comprises the incubation of serum, tissue or body fluids with antibodies against UreX or UreY polypeptide or an antigenic fragment thereof according to the invention.

Exampl 1

The *ure*X and *ure*Y genes of *Helicobacter felis* strain CS1: cloning and expression in *Escherichia coli*.

The *ure*X and *ure*Y genes of *H. felis* strain CS1 were cloned as an operon into an *E. coli* T7 expression vector, pET3a, as follows:

For proper expression of the UreX and Y proteins in pET3a (Novagen, 601 Science Drive, Madison WI, USA), the genes were cloned as a *Nde*I-*Bam*HI DNA fragment into the *Nde*I-*Bam*HI sites of this vector. The *ureaseXY* operon contains an internal *Nde*I site that was mutated by overlap-extension PCR of 2 PCR fragments. For that purpose two PCR fragments (the 5' and the 3' products) were amplified using chromosomal DNA of H. *felis* CS1 as the template. The 5' PCR product contained the complete *ure*X gene and the first part of the *ure*Y gene. The forward primer contained a *Nde*I restriction site and the start codon of ureX (GGAGTAACATATGAAACTCACA CCCAAAGAGC) (SEQ ID NO: 18), and the reverse primer contains a point mutation (CACACCC ACGACCATGTGAGGGCTTAC) (SEQ ID NO: 19). The second 3' PCR product consisted of the 3' end of the *ure*Y gene. This forward primer is complementary to the reverse primer of the first PCR product and also contained the same point mutation (GTAAGCC CTCACATGGTCGTGGGTGTG) (SEQ ID NO: 20), and the reverse primer contained a *Bam*HI restriction site just downstream of the stop codon of the *ure*Y gene (CGAATT CGGATCCTAGAAGAAAGTGTAGCGCTGG) (SEQ ID NO: 21). The mutation in the complementary primers is made to delete the internal *Nde*I site in *ure*Y, it replaces the CATATG (His- Met) by CACATG (His-Met).

After amplification of both PCR products, the complete operon was obtained by overlap-extension-PCR with the forward primer of the *ure*X and the reverse primer of the *ure*Y using both PCR products as templates. The resulting PCR product was cloned into PCR-bluntII-TOPO (Invitrogen, P.O.Box 2312, 9704 CH Groningen, The Netherlands) and transformed into *E. coli* TOP10F' cells (Invitrogen). Positive clones were isolated and the *urease*XY genes were sub-cloned into pET3a with *Nde*I-*Bam*HI. The obtained plasmid was called pUreXY-1 and was transformed into the expression strain HMS174(DE3)/pLysS (Novagen).

The *ure*X and *ure*Y genes of pUreXY-1 were expressed in HMS174(DE3)/pLysS as follows: an overnight culture was diluted 1/100 into TB Amp^{100} Cam^{25}; this culture was incubated for 3 h at 37°C at 200 rpm; the culture was induced by adding 1 mM of IPTG and incubated for another 3 h at 37°C at 200 rpm. The induction was done twice, once in a small scale and once in a large scale.

The induced samples were analysed on a SDS-PAGE gel (fig. 2). As can be clearly seen from lane 9, expression of UreX and UreY, when induced provides the two structural subunits as polypeptide bands with a molecular weight of 25 kDa for the UreX subunit and 62 kDa for the UreY subunit.

Legend to the figures ~~Brief Summary of the Drawings~~

Figure 1a: Comparison of the nucleic acid sequence encoding UreX and Y, including a short non-coding region bridging the two coding sequences, from *Helicobacter felis* species CS1, Kukka, Ds4, 2301 and 390 with the nucleic acid sequence encoding UreA and B, including a short non-coding region bridging the two coding sequences, from *Helicobacter felis*, *pylori* and *heilmannii*

Figure 1b: Comparison of the amino acid sequence of UreX from *Helicobacter felis* species CS1, Kukka, Ds4, 2301 and 390 with the amino acid sequence encoding UreA from *Helicobacter felis*, *pylori* and *heilmannii*

Figure 1c: Comparison of the amino acid sequence of UreY from *Helicobacter felis* species CS1, Kukka, Ds4, 2301 and 390 with the amino acid sequence encoding UreB from *Helicobacter felis*, *pylori* and *heilmannii*

Figure 2: Polyacrylamide gel of the expression products UreX and UreY

Lane 7	: Biorad broad range marker
Lane 8	: Complete cell culture before induction (small scale culture)
Lane 9	: Complete cell culture after induction (small scale culture)
Lane 10	: Complete cell culture after induction (large scale culture)
Lane 11	: Supernatant after induction (large scale culture).
Lane 12	: Biorad pre-stained marker

Amend p. 1

A

Abstract

The present invention relates to novel *Helicobacter felis* urease subunit polypeptides and to nucleic acid sequences encoding these subunit polypeptides, to DNA fragments and recombinant DNA molecules comprising the nucleic acid sequences encoding these subunit polypeptides, to live recombinant carriers and to host cells comprising nucleic acid sequences encoding these subunit polypeptides. Also, the invention relates to the subunit polypeptides for use in vaccines and the use in the manufacturing thereof, to vaccines comprising said subunit polypeptides and to methods for the preparation of such vaccines. Furthermore, the invention relates to diagnostic methods for the detection of *Helicobacter felis* specific nucleic acid sequences, *Helicobacter felis* antigenic material and to antibodies against *Helicobacter felis*.